STANDARD OPERATING PROCEDURE FOR ALKYLPHENOLS USING LC-MS/MS

ECB-010.0

July 2012

United States Environmental Protection Agency

Environmental Sciences Division

Environmental Chemistry Branch

7-19-12

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7/19/12

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STANDARD OPERATING PROCEDURE FOR ANALYZING ALKYLPHENOLS USING LC-MS/MS

1.0 Disclaimer

This standard operating procedure has been prepared for use of the Environmental Sciences Division, Environmental Chemistry Branch, NERL, ORD of the U.S. Environmental Protection Agency and may not be specifically applicable to the activities of other organizations. **THIS IS NOT AN OFFICIAL EPA APPROVED METHOD.** This document has not been through the Agency's peer review process or ORD clearance process.

2.0 Purpose (Scope and Application)

This document describes the procedure for the determination of alkylphenols (APs), including octylphenol (OP) and nonylphenol (NP), in aqueous samples by solid-phase extraction (SPE) and liquid chromatography-mass spectrometry (LC-MS/MS) using a triple quadrupole mass spectrometer.

3.0 Method Summary

3.1 The method employs high-performance liquid chromatography (HPLC) coupled with negative electrospray ionization (ESI-) tandem mass spectrometry (MS/MS) for the determination of APs in aqueous matrices.

3.2 Aqueous samples are first flowed through Oasis HLB SPE cartridges (polystyrene-divinylbenzene-N-vinylpyrrolidone terpolymer resin) to extract the ethoxylated compounds from solution before concentrating the samples to 0.5 mL.

3.3 Target compounds are identified by retention time and multiple-reaction monitoring (MRM) transition using MS/MS. Compounds are quantified using internal standards.

4.0 Interferences

4.1 All glassware must be washed with detergents free from alkylphenol ethoxylates. Powdered Alconox does not contain ethoxylated alcohols, but any comparable detergent free from these interferences may also be used.

4.2 Method interferences can be caused by contaminants in glassware, solvents, and other apparatus producing discrete artifacts or elevated baselines. These materials are routinely demonstrated to be free from interferences by analyzing laboratory reagent blanks and method blanks under the same conditions as the samples.

4.3 Matrix interferences may be caused by contaminants that are co-extracted from the sample.

4.4 The Oasis HLB cartridges have been known to be contaminated with NP and OP. Proper conditioning of cartridges with isopropanol and methanol is very crucial to ensure that APs are not found in cartridges.

5.0 Safety

5.1 All of the chemicals used in this procedure should be handled only while using proper personal protective equipment such as gloves, lab coats, safety glasses and fume hoods. The analyst should review the Material Safety Data Sheet for each chemical in this procedure so that safe working conditions can be achieved. NP is a known endocrine-disrupting compound.

5.2 The toxicity of each reagent used in this method may not have been fully established. Each chemical should be regarded as a potential health hazard, and exposure should be kept as low as reasonably achievable.

5.3 Waste must be disposed of in appropriate waste containers. Contact the onsite SHEM Program Manager to dispose of full waste containers.

5.4 Exhaust fumes from the HPLC-MS must be properly vented.

5.5 All applicable safety and compliance guidelines set forth by the EPA and by federal, state, and local regulations must be followed during the performance of this SOP. Stop all work in the event of a known or potential compromise to the health and safety of any person and immediately notify the SHEM Program Manager and other appropriate personnel.

5.6 Analysts must be cognizant of all instrumental hazards (i.e., dangers from electrical shock, heat, or explosion).

6.0 Reagents/Chemicals/Gases

6.1 HPLC-grade Methanol

6.2 HPLC-grade Water

6.3 Deionized (DI) water: in-house 18 MΩ-cm DI water

6.4 Methyl t-butyl ether (MTBE)

6.5 AP standards. NP and OP.

6.5.1 NP is a mixture of branched p-NP isomers. Commercial NP is produced by the reaction of phenol with commercial nonene. Commercial nonene is not simply a linear C_9H_{18} alpha olefin (i.e., n-nonene), instead it is a complex mixture of predominantly nine-carbon olefins, called propylene trimer, containing no linear isomers. This synthesis results in a mixture of various branched nonylphenol isomers rather than a discrete chemical structure. The branched nonyl group is positioned predominantly in the *para* position on the phenol ring.

6.5.2 Commercial OP is produced by the reaction of phenol and diisobutylene to produce predominantly the 4-(1,1,3,3-tetramethylbutyl)phenol isomer.

6.6 Deuterated OP (OP-d2): 4-tert-octylphenol-3,5-d2 (Sigma-Aldrich) 1 ug/mL in acetone.

6.7 Isopropyl alcohol

6.8 Ammonium acetate

6.9 Polypropylene glycol standard from AB SCIEX

7.0 Equipment and Supplies

7.1 HPLC-MS system: (AB Sciex QTRAP 4000 coupled with Shimadzu LC-20AD HPLC). Data acquisition software Analyst 1.5.

7.2 HPLC column (Acquity UPLC BEH C18 1.7 µm, 2.1 x 100 mm). Other columns may be used if they provide sufficient retention and separation of the target analytes.

7.3 Variable volume standard pipettors (0.5 -10 µL, 20-200 µL, 100-1000 µL)

7.4 Pipet tips

7.5 Glass beakers, volumetric flasks, sized as appropriate

7.6 Disposable borosilicate Pasteur pipets

7.7 Ultra-high-purity grade compressed nitrogen

7.8 1 mL autosampler vials with PTFE/silicone septa

7.9 Disposable 0.45 µm syringe tip filters, if needed to remove suspended solids

7.10 Filtering apparatus for filtering large volume samples using glass fiber filter discs (type 934-AH, or equivalent), if necessary

7.11 TurboVap Concetrator, for concentrating samples

7.12 Autotrace SPE Workstation

7.13 Oasis HLB SPE cartridges (200 mg, 6 cc size)

8.0 Sample Collection, Preservation, and Storage

8.1 This SOP does not describe sample collection procedures; however, the following guidelines are followed once samples are received in the laboratory.

8.2 Samples must be stored at 4°C in a designated sample refrigerator.

8.3 Holding time studies have not been performed on these analytes; however, samples should be analyzed as soon as possible, and within 28 days.

9.0 Quality Control

9.1 The following are relevant QC criteria for this method.

Table 1. Data Quality Indicators of Measurement Data.

QC Check	Frequency	Completeness	Precision	Accuracy	Corrective Action
Initial 5-point calibration	Prior to sample analysis	100%	RSD≤20%	$R^2 > 0.99$	No samples will be run until calibration passes criteria.
Laboratory blank	One per batch of samples[a]	100%	N/A	< PQL[b]	Inspect the system and reanalyze the blank. Samples must be bracketed by acceptable QC or they will be invalidated.
Instrument blank	One at beginning of each 8-hr analytical day, one at beginning of each batch of samples[a], and one at end of analytical day	100%	N/A	< PQL[b]	Inspect the system and reanalyze the blank. Samples must be bracketed by acceptable QC or they will be invalidated.
Laboratory control sample (LCS)	One per batch of samples[a]	100%	RPD≤30%[c]	± 30% of known value	Check the system and reanalyze the standard. Re-prepare the standard if necessary. Recalibrate the instrument if the criteria cannot be met. Samples must be bracketed by acceptable QC or they will be invalidated.

Laboratory fortified matrix	One per batch of samples[a]	100%	RPD≤30%[c]	>60% recovery	Review data to determine whether matrix interference is present. If so, narrate interference and flag recovery. If no interference is evident, verify the instrument is functioning properly by running a lab blank. Reanalyze recollected sample to verify recovery. Samples must be bracketed by acceptable QC or they will be invalidated.
Laboratory replicates	One per batch of samples[a]	100%	RPD≤30%[c]	>60% recovery	Inspect the system, narrate discrepancy. Samples must be bracketed by acceptable QC or they will be invalidated.
Continuing calibration verification (CCV)	One at beginning of each 8-hr analytical day, one at beginning of each batch of samples[a], and one at end of analytical day	100%	RSD≤30%[c]	± 30% of known value	Inspect system and perform maintenance as needed. If system still fails CCV, perform a new 5-point calibration curve. Samples must be bracketed by acceptable QC or they will be invalidated.
Laboratory fortified blank	One per batch of samples[a]	100%	N/A	>60% recovery	Inspect the system and reanalyze the standard. Re-prepare the standard if necessary. Re-calibrate the instrument if the criteria cannot be met. Samples must be bracketed by acceptable QC or they will be invalidated.
Minimum detection limit	Each chemical	100%	TBD for each HF chemical	TBD for each HF chemical	TBD for each HF chemical

[a]Batch of samples not to exceed 20
[b]PQL=practical quantitation limit, 5 times the MDL
[c]Precision among replicates if more that 1 batch of samples are analyzed. RSD may be applicable if more than 2 replicates are analyzed.

10.0 Calibration and Standardization

10.1 Tune and calibrate MS (both Q1 and Q3) according to manufacturer's directions using the AB Sciex PPG standard (diluted 50:1 with 50/50 water/methanol containing 0.1% formic acid and 2 mM ammonium acetate).

10.3 Tuning to determine the correct system settings (e.g., curtain gas, temperature, IonSpray voltage, declustering potential, etc.) for particular analytes is performed as needed and according to the manufacturer's directions. This is done according to the manufacturer's instructions using the Compound Optimization feature in the Analyst software. Representative settings for the analytes in this method are listed in section 11.4.

10.4 Record all instrument maintenance in the instrument maintenance log book.

10.5 Suggested concentrations for the initial AP calibration levels are 10 to 800 ppb OP and NP, each containing a constant concentration of 200 ppb OP-d2. A minimum of 5 calibration levels must be used.

10.6 Calibration by isotope dilution: isotope dilution is used for calibration of each native compound for which a labeled analog is available. In this case, that corresponds to OP (Note: deuterated analogs of NP are available, but only in the n-NP form. The n-NP has considerably different extraction recoveries than the technical mixture of NP branched isomers). For NP, calibration is done using OP-d2 as the internal standard.

10.6.1 To calibrate the system by isotope dilution, inject the calibration standards (minimum of 5).

10.6.2 For the compounds determined by isotope dilution, the relative response (RR) (labeled to native) vs. concentration in the calibration solutions is computed over the calibration range according to the procedure below. Determine the response of each compound relative to its labeled analog using the area responses of the Q3 ions specified in section 11.4.3.

10.6.3 Compute the native compounds with a labeled analog using the following equation:

$$RR = \frac{A_n C_l}{A_l C_n}$$

Where:

A_n= Area of Q3 mass of native compound
A_l= Area of Q3 mass of labeled compound
C_l= Concentration of labeled compound in the calibration standard
C_n=Concentration of native compound in the calibration standard.

10.6.4 Compute the average (mean) RR, and the standard deviation and relative standard deviation (RSD) of the RRs.

10.6.5 Linearity: If the RR for any compound is constant (less than 20% RSD), the average RR may be used for that compound; otherwise, the complete calibration curve for that compound must be used over the calibration range.

10.7 Calibration by internal standard: internal standard calibration is applied to the determination of the native compounds for which a labeled compound is not available.

10.7.1 Response factors: internal standard calibration requires the determination of response factors (RF) defined by the following equation:

$$RF = \frac{A_n C_{is}}{A_{is} C_n}$$

Where:

A_n= Area of Q3 mass of native compound
A_{is}= Area of Q3 mass of internal standard
C_{is}= Concentration of internal standard in the calibration standard
C_n=Concentration of native compound in the calibration standard.

10.7.2 Linearity: if the RF for the native compound without labeled analog is constant (less than 30% RSD), the average RF may be used for that compound; otherwise, the complete calibration curve must be used over the calibration range.

11.0 Procedure

11.1 Glassware cleaning

11.1.1 Prepare soapy bath with hot water and approximately 1 tsp Alconox detergent. Scrub glassware with bottle brushes and/or pipe cleaners until visibly clean (do not scratch glassware with metal from brushes). Rinse glassware first with non-DI water, and then with DI water. Soak glassware in acid bath (3 mL HCl, 3 mL HNO_3, 4 L water, pH 1-2) overnight. Remove glassware and rinse with Ultrapure DI water. Rinse glassware with methanol and air dry. Place glassware in oven at 100°C for 6 hours.

11.2 Sample preparation

11.2.1 Add an appropriate amount of internal standard (OP-d2) to a known volume of aqueous sample for extraction. For the purposes of this method, the addition of 250 ng of OP-d2 to 500 mL sample worked well.

11.2.2 If aqueous sample contains suspended solids, first filter using filtering apparatus and glass fiber discs. Ensure that the glass fiber disc is rinsed well with water, followed by MTBE.

11.3 Solid-phase extraction

11.3.1 Precondition the Oasis HLB cartridges by loading 20 mL isopropyl alcohol through each cartridge. Discard isopropyl alcohol.

11.3.2 Load cartridges into Autotrace SPE Workstation, and condition the cartridges with 5 mL methanol, followed by 5 mL water, both at a flow rate of 5 mL/min.

11.3.3 Load 500 mL aqueous sample through the SPE cartridges at a flow rate of 5 mL/min. Rinse the volumetric flasks with 50 mL water, and load this water through the SPE cartridges as well.

11.3.4 Rinse the cartridges with 2 mL water at a flow rate of 3 mL/min, and dry the cartridges with N_2 for 30 min.

11.3.5 Elute with 5 mL 2:2:1 methanol/acetone/ethyl acetate and then with 5 mL 90:10 MTBE/methanol, both at a flow rate of 1 mL/min.

11.3.6 Quantitatively transfer the eluate from the Autotrace collection tube to a TurboVap tube. Concentrate and solvent exchange the eluate into methanol using the TurboVap Concentrator. Concentrate to 0.5 mL methanol.

11.3.7 Transfer the concentrated sample with Pasteur pipet to an autosampler vial.

11.3.8 Filter the samples, if necessary, with a syringe filter prior to MS analysis.

11.4 LC-MS analysis

11.4.1 Mobile phase A consists of 2 mM ammonium acetate in HPLC grade water. Mobile phase B consists of 2 mM ammonium acetate in HPLC grade acetonitrile.

11.4.2 The following LC gradient is used to analyze alkylphenols (column temperature of 30°C):

Time (min)	Flow rate (mL/min)	%A	%B
Initial	0.25	60	40
2	0.25	60	40
8	0.25	5	95
10	0.25	5	95
12.5	0.25	60	40
20	0.25	60	40

11.4.3 MS analysis conditions: Source conditions: Curtain gas: 25, IonSpray Voltage: -4500, Temperature: 200, Ion Source Gas 1: 90, Ion Source Gas 2: 50, Collision Gas: 9, Needle position: x=5.50, y= 2.00.

Compound	Q1 Mass	Q3 Mass	Dwell Time (msec)	DP	CE	CXP	EP
NP	219.2	133	400	-90	-36	-9	-11
OP	205.2	133	200	-90	-34	-7	-11
OP-d2	207.2	135	200	-80	-33	-6	-11

DP=declustering potential
CE=collision energy
CXP=collision exit potential
EP=entrance potential

11.4.4 Load samples into the Shimadzu SIL-20AC autosampler. In the Analyst 1.5 software, select Build Acquisition Batch under "Acquire" to build a new sample batch. Click "Add Set", and then "Add Samples". Enter an appropriate sample prefix name and a data file prefix, and type in the number of samples to be added, then click "OK". Right-click on the sample batch table, and select "Hide/Show Column". Place a checkmark next to "Sample ID", and click "OK". In the sample batch, enter appropriate sample IDs, vial numbers, an injection volumes. Select the appropriate acquisition method. Click on the "Submit" tab, and then select the "Submit" button. This action uploads the samples into the Acquisition Queue.

11.4.5 Select "View->Sample Queue" to progress to the sample queue page. Select "Acquire->Equilibrate". Select the appropriate acquisition method, enter a time of 30 min, and click "OK". This equilibrates the MS and LC conditions for a set amount of time.

11.4.6 Ensure that LC solvent levels are adequate and that there is enough N_2 gas to complete the runs. Once the instrument is ready after equilibration (under Queue Server, the icon will say "Ready"), select "Acquire"->Start Sample". This begins the sample acquisition process.

11.4.7 The instrument will automatically go into "Standby" mode after the sample queue completes.

11.5 Data Analysis

11.5.1 In the Analyst software, select "Quantitation Wizard" under the "Quantitate" menu item in the left-hand side of the screen. Select the appropriate data files, and add them to the right-hand "Selected Samples" window. Click "Next" twice, and then choose an appropriate quantitation method to use. Click "Finish".

11.5.2 After running the Quantitation Wizard, inspect each chromatographic peak to ensure that the peak has been integrated properly. The peaks may not be Gaussian in appearance due to the presence of multiple isomers.

11.5.3 Identify and confirm the presence of target analytes in the samples by matching the retention times of the MRMs. The retention time window of the analytes must be within 10% of the retention time of the analyte in the midpoint calibration standard.

11.5.4 Quantitate the amounts of each analyte using isotope dilution or internal standard techniques. By adding a known amount of a labeled compound to every sample prior to extraction, correction for recovery of the native analog of that compound can be made because the native compound and its labeled analog exhibit similar chemical properties upon extraction, concentration, and chromatography.

11.5.4.1 Compute the concentration of each native compound that has a labeled analog in the extract using the RR from the calibration data (section 10.6) and following equation:

$$C_{ex} = \frac{A_n C_l}{A_l RR}$$

Where: C_{ex}= concentration of compound in the extract, and the other terms are defined in section 10.6.

11.5.4.2 Compute the concentration of each native compound that does not have a labeled analog using the RF from the calibration data (section 10.7) and the following equation:

$$C_{ex} = \frac{A_n C_{is}}{A_{is} RF}$$

Where: C_{ex}= concentration of compound in the extract, and the other terms are defined in section 10.7.

11.5.5 Calculate the extraction recovery based on the recovery of the surrogate:

$$\%R = 100\% \times \frac{C_m}{C_{srm}}$$

Where:
%R = percent recovery of labeled analog
C_m= measured concentration of standard reference material
C_{srm} = actual concentration of standard reference material

Note: During calculations, take into account the concentration factor from the 500 mL sample down to 0.5 mL following extraction/concentration.

11.5.7 Calculate the spike recoveries:

$$\%\mathrm{R} = 100\% \times \frac{(S - U)}{C_{sa}}$$

Where:
%R = percent recovery
S = measured concentration in spiked aliquot
U = measured concentration in unspiked aliquot
C_{sa} = actual concentration of spike added

12.0 Method Performance

12.1 Method performance is evaluated based on the criteria in Table 1.

12.2 MDLs have not been determined for these compounds yet. Reporting limits are 0.05 µg/L for OP and NP.

13.0 References

ASTM D 7485-09. "Standard Test Method for Determination of Nonylphenol, p-tert-Octylphenol, Nonylphenol Monoethoxylate and Nonylphenol Diethoxylate in Environmental Waters by Liquid Chromatography/Tandem Mass Spectrometry", 2009.

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